An ancient founder mutation in PROKR2 impairs human reproduction

Avbelj Stefanija, Magdalena ; Jeanpierre, Marc ; Sykiotis, Gerasimos P ; Young, Jacques ; Quinton, Richard ; Abreu, Ana Paula ; Plummer, Lacey ; Au, Margaret G ; Balasubramanian, Ravikumar ; Dwyer, Andrew A ; Florez, Jose C ; Cheetham, Timothy ; Pearce, Simon H ; Purushothaman, Radhika ; Schinzel, Albert ; Pugeat, Michel ; Jacobson-Dickman, Elka E ; Ten, Svetlana ; Latronico, Ana Claudia ; Gusella, James F ; Dode, Catherine ; Crowley, William F ; Pitteloud, Nelly

Abstract: Congenital gonadotropin-releasing hormone (GnRH) deficiency manifests as absent or incomplete sexual maturation and infertility. Although the disease exhibits marked locus and allelic heterogeneity, with the causal mutations being both rare and private, one causal mutation in the prokineticin receptor, PROKR2 L173R, appears unusually prevalent among GnRH-deficient patients of diverse geographic and ethnic origins. To track the genetic ancestry of PROKR2 L173R, haplotype mapping was performed in 22 unrelated patients with GnRH deficiency carrying L173R and their 30 first-degree relatives. The mutation's age was estimated using a haplotype-decay model. Thirteen subjects were informative and in all of them the mutation was present on the same 123 kb haplotype whose population frequency is 10%. Thus, PROKR2 L173R represents a founder mutation whose age is estimated at approximately 9000 years. Inheritance of PROKR2 L173R-associated GnRH deficiency was complex with highly variable penetrance among carriers, influenced by additional mutations in the other PROKR2 allele (recessive inheritance) or another gene (digenicity). The paradoxical identification of an ancient founder mutation that impairs reproduction has intriguing implications for the inheritance mechanisms of PROKR2 L173R-associated GnRH deficiency and for the relevant processes of evolutionary selection, including potential selective advantages of mutation carriers in genes affecting reproduction.

DOI: https://doi.org/10.1093/hmg/dds264

An Ancient Founder Mutation in *PROKR2* Impairs Human Reproduction

Magdalena Avbelj,[1,2] Marc Jeanpierre,[3] Gerasimos P. Sykiotis,[4] Jacques Young,[5] Richard Quinton,[6,7] Ana Paula Abreu,[8] Lacey Plummer,[1] Margaret G. Au,[1] Ravikumar Balasubramanian,[1] Andrew A. Dwyer,[9] Jose C. Florez,[10,11,12] Timothy Cheetham,[6,13] Simon H. Pearce,[6] Radhika Purushothaman,[14] Albert Schinzel,[15] Michel Pugeat, [16] Elka E. Jacobson-Dickman, [14] Svetlana Ten,[14] Ana Claudia Latronico,[8] James F. Gusella,[11,17] Catherine Dode,[3] William F. Crowley Jr., [1,12] Nelly Pitteloud[9]

[1] Harvard Reproductive Endocrine Sciences Center and the Reproductive Endocrine Unit of the Department of Medicine, Massachusetts General Hospital, 02114 Boston, Massachusetts;
[2] Department of Pediatric Endocrinology, Diabetes and Metabolism at University Children's Hospital, University Medical Centre Ljubljana, 1000 Ljubljana, Slovenia;
[3] Institut Cochin, Université Paris Descartes, INSERM U1016, 75014 Paris, France;
[4] Department of Internal Medicine, Division of endocrinology and Department of Pharmacology, University of Patras Medical School, 26500 Patras, Greece;
[5] Université Paris-Sud 11 et INSERM U693, Faculté de Médecine Paris Sud, 94276 Le Kremlin Bicêtre, France;
[6] Institute for Human Genetics, Newcastle University, NE1 3BZ Newcastle upon Tyne, United Kingdom;
[7] Department of Endocrinology, Newcastle upon Tyne Hospitals, NE1 7RU Newcastle upon Tyne, United Kingdom;
[8] Unidade de Endocrinologia do Desenvolvimento, Laboratório de Hormônios e Genética Molecular, LIM/42, Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, 05403-900 São Paulo, Brasil;
[9] Endocrine, Diabetes, & Metabolism Service, Centre Hospitalier Universitaire Vaudois (CHUV), 1011 Lausanne, Switzerland ;
[10] Center for Human Genetic Research and Diabetes Research Center (Diabetes Unit), Department of Medicine, Massachusetts General Hospital, 02114 Boston, Massachusetts;
[11] Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, 02142 Cambridge, Massachusetts;
[12] Department of Medicine, Harvard Medical School, 02114 Boston, Massachusetts;
[13] Department of Paediatrics, Newcastle upon Tyne Hospitals, NE1 4LP Newcastle upon Tyne, United Kingdom;
[14] Divisions of Pediatric Endocrinology at Maimonides Infants and Children's Hospital of Brooklyn and SUNY Downstate Medical Center, NY 11219 Brooklyn, New York;
[15] Institute of Medical Genetics, University of Zürich, CH-8603 Schwerzenbach, Zürich, Switzerland;
[16] INSERM U1060, Université Lyon 1, and Fédération d'Endocrinologie, Hospices Civils de Lyon, F-69677 Bron Cedex, France;

[17] Center for Human Genetic Research, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, 02114 Boston, Massachusetts.

Correspondence author:
Prof. Nelly Pitteloud, MD,
Endocrine, Diabetes, & Metabolism Service
Centre Hospitalier Universitaire Vaudois (CHUV)
Rue du Bugnon 46, BH 19-701
CH-1011 Lausanne (Switzerland)
Phone: +41 21 314 o6 00
Fax: +41 21 314 06 30
Nelly.Pitteloud@chuv.ch

ABSTRACT

Congenital gonadotropin-releasing hormone (GnRH) deficiency manifests as absent or incomplete sexual maturation and infertility. Although the disease exhibits marked locus and allelic heterogeneity, with the causal mutations being both rare and private, one causal mutation in the prokineticin receptor, *PROKR2* L173R, appears unusually prevalent among GnRH-deficient patients of diverse geographic and ethnic origins. To track the genetic ancestry of *PROKR2* L173R, haplotype mapping was performed in 22 unrelated patients with GnRH deficiency carrying L173R and their 30 first-degree relatives. The mutation's age was estimated using a haplotype-decay model. Thirteen subjects were informative and in all of them the mutation was present on the same ~123 kb haplotype whose population frequency is ≤10%. Thus, *PROKR2* L173R represents a founder mutation whose age is estimated at ~9,000 years. Inheritance of *PROKR2* L173R-associated GnRH deficiency was complex with highly variable penetrance among carriers, influenced by additional mutations in the other *PROKR2* allele (recessive inheritance) or another gene (digenicity). The paradoxical identification of an ancient founder mutation that impairs reproduction has intriguing implications for the inheritance mechanisms of *PROKR2* L173R-associated GnRH deficiency and for the relevant processes of evolutionary selection, including potential selective advantages of mutation carriers in genes affecting reproduction.

INTRODUCTION

Mammalian reproduction is controlled at the neuroendocrine level by the tightly coordinated pulsatile secretion of gonadotropin-releasing hormone (GnRH) from a small network of ~ 1,200 specialized hypothalamic neurons (1, 2). GnRH secretion stimulates the anterior pituitary to release gonadotropins which, in turn, act on the gonads to regulate steroidogenesis and gametogenesis. Corresponding to the dominating role of this single hormone in the hypothalamic-pituitary-gonadal axis, the developmental ontogeny and homeostatic regulation of the GnRH neuronal network is highly complex and tightly linked to evolutionary fitness. Unique insights into the genetic networks and signaling pathways involved in GnRH neuronal ontogeny and hormonal action have been derived from the study of the human disease model of congenital isolated GnRH deficiency (3). This genetic disease manifests as incomplete or absent sexual maturation and infertility, and it is clinically diagnosed as idiopathic hypogonadotropic hypogonadism (IHH [MIM 146110]) with either normal sense of smell (normosmic IHH, nIHH) or anosmia (Kallmann syndrome, KS [MIM 147950])(4). Although the current knowledge of GnRH biology is incomplete, it is evident that multiple developmental and neuroendocrine pathways are involved in the ontogeny and homeostasis of GnRH neurons, the secretion of GnRH, and its action on the pituitary. This complex regulation likely facilitates adaptation to diverse environmental pressures on reproduction to ensure survival and evolution of the species.

The chemokine prokineticin 2 (PROK2) and its cognate G protein-coupled receptor (PROKR2) comprise one of the major signaling systems involved in GnRH ontogeny.

The mammalian prokineticin family has been implicated in diverse biologic processes including neurogenesis, angiogenesis, carcinogenesis, circadian rhythm regulation, inflammation, immune system modulation, and pain perception (5). In the mouse brain Prokr2 is the predominant prokineticin receptor, with high expression in the olfactory bulb and hypothalamus (6-8). During embryonic development Prok2-Prokr2 signaling is essential for neurogenesis in the olfactory bulb and for the migration of GnRH neurons from their developmental origin in the olfactory placode to the hypothalamus (8-10). *Prok2*$^{-/-}$ and *Prokr2*$^{-/-}$ mice display hypoplastic olfactory bulbs, disrupted GnRH neuronal migration, and hypoplastic reproductive organs due to hypogonadotropic hypogonadism (9, 10). Consistently, germline loss-of-function mutations in *PROK2* (MIM 607002) and *PROKR2* (MIM 607123) have been identified in patients with isolated GnRH deficiency (11-17).

Without hormonal treatments to induce puberty and confer reproductive capacity, the large majority of IHH patients are infertile. Therefore, the causative mutations must have been subjected to strong purifying (i.e., negative) selection during evolution. In accordance with this notion, almost every mutation that has been shown to underlie GnRH deficiency is rare among patients, and many mutations are even unique to specific patients or families (17). However, a few mutations occur in strikingly high percentages of GnRH-deficient patients who harbor mutations in the respective gene, including *GNRHR* (MIM 138850) Q106R (44%), *GNRHR* R262Q (29%) (17-23), and *TACR3* (MIM 162332) W275X (36%) (24-26). In the prokineticin pathway, a commonly reported mutation *PROKR2* L173R (40%) has emerged (11-13, 15-17). In addition to its

implication in congenital isolated GnRH deficiency, the same mutation was also associated with hypothalamic amenorrhea (HA)(27), a common reproductive disorder in females characterized by a reversible form of GnRH deficiency triggered by stressors.

The causal role of *PROKR2* L173R in GnRH deficiency is strongly supported by the demonstration of dramatic reduction of the mutant receptor's activity in cell-based functional assays (12, 28). Furthermore, homozygous patients phenocopy *Prokr2*$^{-/-}$ mice by presenting with severe KS (10, 11, 16). Notably, the *PROKR2* L173R mutation has been identified in numerous patients with congenital GnRH deficiency from diverse geographic locations and ethnic backgrounds (11-13, 15-17). This could be either because the mutation has arisen *de novo* multiple times during human evolution (i.e., it is a "hot spot" mutation), or because it has been inherited from a common ancestor (i.e., it is a "founder" mutation) despite being associated with infertility. To distinguish between these possibilities, this study tracked the genetic ancestry of *PROKR2* L173R; it also established the mutation's frequency among large cohorts of patients with congenital GnRH deficiency and unaffected controls from various geographic and ethnic populations, and determined the inheritance patterns of GnRH deficiency in mutation carriers. Having documented that it is a founder mutation, its age was estimated; and lastly, a plausible explanation for the persistence of the mutation in the human gene pool is offered.

RESULTS

PROKR2 L173R is a founder mutation of ancient origin

The *PROKR2* haplotype was evaluated in 23 probands (including 22 patients with GnRH deficiency and 1 proband with HA) carrying the *PROKR2* L173R mutation and 33 available family members by genotyping 5 flanking short tandem repeats (STRs) and 93 single nucleotide polymorphisms (SNPs). In 13 probands (11 heterozygotes and 2 homozygotes) the haplotypes at the *PROKR2* locus could be unambiguously determined (Supplemental Table 1). In all of these probands *PROKR2* L173R resided on the same core haplotype which has a size of ~123 kb (Figure 1). In the remaining 10 probands, the haplotypes at the *PROKR2* locus could not be unambiguously determined; nevertheless, the presence of *PROKR2* L173R on the same haplotype was plausible (Supplemental Table 1). This shared haplotype does not extend beyond major recombination hot spots flanking *PROKR2* and does not include adjacent genes, making their contribution to this disease highly unlikely. According to HapMap Project data, the frequency of the shared haplotype is 6.7% in Europeans (Utah residents with Northern and Western European ancestry), <1% in Africans (Yoruba from Ibadan, Nigeria), and 2.2% in East Asians (Han Chinese from Beijing and Japanese from Tokyo) (29). Thus, on the assumption that the frequency of the haplotype in the general population is not higher than 10%, the probability that *PROKR2* L173R is a hot spot mutation that arose *de novo* 15 independent times (including 11 possibly independent events in 11 heterozygots and 4 in 2 homozygots) on the same haplotype is $\leq 10^{-15}$. Therefore, *PROKR2* L173R in all likelihood represents a founder mutation. The small size of the shared haplotype and the evidence of multiple recombination events on each flank (Figure 1) indicate that the

genotyped families are branches of a very ancient genealogy. Based upon the size of the shared haplotype and the shape of the genetic tree, the age of the *PROKR2* L173R mutation is estimated by an algorhitm (30) to be ~300 generations (~9,000 years) with a 95% confidence interval of approximately 150-400 generations. Because nearly all branches of the genetic tree have distinct ends, a biased result from a putative recombination hot-spot can be ruled out, and the estimation of the mutation's age is robust. The mutation thus likely arose during the Neolithic expansion of the human population that happened ~5,500-12,000 years ago.

Hypothalamic amenorrhea-associated *PROKR2* L173R shares the same ancient haplotype

The *PROKR2* locus haplotype of the Caucasian proband (#6) with hypothalamic amenorrhea, a milder form of GnRH deficiency, and 3 of her first-degree relatives were studied. The mutation was found to reside on the same core haplotype as in the probands with congenital GnRH deficiency (Figure 1). The patient's affected son with delayed puberty and her asymptomatic daughter had both inherited the disease haplotype (Figure 2).

***PROKR2* L173R is one of the most common mutations in congenital GnRH deficiency**

As an indicator of the strength of evolutionary selective pressure on an ancient founder mutation, the frequency of the *PROKR2* L173R mutation was evaluated in a total of 1,299 GnRH deficient patients and 1214 unaffected ethnically matched controls. The

PROKR2 L173R mutation was identified in 2.4% (minor allele frequency (MAF)=1.3%) of unrelated patients of diverse ethnic origins, including Caucasians, Brazilians, Mexicans, and Maghrebis (inhabitants of a large region in North Africa stretching from Morocco to Libya) (Table 1). Among the unaffected control subjects, the *PROKR2* L173R mutation was identified only in two Caucasian females with otherwise normal puberty and proven fertility (carrier rate=0.16%, MAF=0.08%). On the other hand, *PROKR2* L173R is one of the most commonly reported mutations associated with isolated GnRH deficiency. In comparison, the most common *GNRHR* mutation, Q106R, was identified in 2.1% of patients (mainly of Caucasian and Brazilian origin) with MAF=1.2% (17, 19-22, 31, 32), and the most common *TACR3* mutation, W275X, was identified in 2.0% of patients screened (MAF=1.4%), with all carriers being Turkish or Kurdish (24-26).

Complex inheritance of congenital GnRH deficiency in *PROKR2* L173R mutation carriers

Isolated GnRH deficiency is increasingly recognized as an oligogenic condition (16, 17, 33). *PROKR2* mutations have been implicated in diverse oligogenic interactions, including mutations in other genes regulating GnRH neuron ontogeny such as *PROK2*, *KAL1*, and *FGFR1* (11, 12, 15-17, 34). The fact that L173R is the most common *PROKR2* mutation facilitates the study of these concepts by reducing complexity due to allelic heterogeneity. Complete penetrance of biallelic *PROKR2* L173R mutations was demonstrated previously in two homozygotes and one compound heterozygote, all of whom had hyposmia or anosmia and severe GnRH deficiency as indicated by absent

pubertal development, microphallus, and/or cryptorchidism (11, 16). Whereas 15/19 probands with monoallelic *PROKR2* L173R mutations also displayed severe GnRH deficiency, two had undergone partial puberty, and one had adult-onset hypogonadotropic hypogonadism (35) that occurred after complete spontaneous pubertal development (Table 2); detailed clinical information on pubertal development was not available for the remaining proband. Of the 19 probands carrying the heterozygous L173R mutation, all but four were hyposmic or anosmic. Of the 18 first-degree family members who harbored the *L173R* mutation only four were affected, demonstrating highly variable penetrance of the heterozygous mutation as evidence by their phenotypes: (i) KS [families 9 (11) &11 (34)], (ii) delayed puberty (family 3), and (iii) borderline sense of smell – 5th percentile (family 11) (Figure 2). In family 3 (Figure 3), the proband and an unaffected sibling carrying the heterozygous *PROKR2* L173R had identical *PROKR2* haplotypes on both paternal and maternal alleles, excluding the possibility that penetrance is determined by additional (non-coding) inherited mutations at the same locus on the non-L173R chromosome and arguing against a recessive mode of disease inheritance due to compound heterozygosity.

Thus far, four cases of digenic inheritance of isolated GnRH deficiency associated with *PROKR2* L173R mutations have been reported, three in combination with *KAL1* mutations (15, 16) and one with an *FGFR1* mutation (34), thus demonstrating that a variety of GnRH deficiency associated loci may influence the penetrance of L173R-associated congenital GnRH deficiency. An additional novel *PROKR2* mutation (M64V) was identified on the same L173R haplotype in proband #4, and a *de novo* chromosomal

translocation was found in proband #5 (36); the functional significance of these two alterations is presently unknown. Most probands harboring the heterozygous *PROKR2* L173R mutation exhibit no additional mutations in either *PROKR2* or other known disease genes. This complex inheritance of GnRH deficiency associated with *PROKR2* L173R suggests a role of yet unknown genetic and/or environmental factors in the pathogenesis and clinical manifestation of the disease.

DISCUSSION

The paradox: A mutation that hinders puberty and reproduction is inherited over thousands of years

Whereas detrimental mutations are progressively eliminated from the human gene pool (37), founder mutations persist and are transmitted to offspring. Because variation in reproductive success is the fundament of natural selection, the existence of founder mutations that compromise pathways involved in the neuroendocrine control of reproduction is at first glance counterintuitive. Nevertheless, such founder mutations have been recently identified in *TAC3* (c.209-1G>C) and *GNRH1* (c.18-19insA) (25, 38). Both of these mutations are exceedingly rare among GnRH-deficient patients (only 3 probands and 1 sibling pair, respectively); have been identified only in specific ethnic groups (Africans from Congo and Haiti; and Romanians, respectively); and have occurred relatively recently (~600 and 240-1500 years ago, respectively) (25, 38). In contrast, *PROKR2* L173R is one of the most commonly identified causative mutations among patients with congenital GnRH deficiency (found in 2.4%) (11-13, 15-17) (Table 1); has been found in patients from diverse ethnic and geographic origins (11-13, 15, 16) (Table

1); and is the oldest known mutation associated with isolated GnRH deficiency with an estimated age of ~9,000 years. Moreover, *PROKR2* L173R is one of the oldest founder mutations impairing reproductive fitness in general, with an age comparable to *CFTR* (MIM 602421) F508del which causes cystic fibrosis (MIM 219700) and arose ~10,000 years ago (39). This highly paradoxical identification of *PROKR2* L173R as an ancient founder mutation has intriguing implications for the relevant processes of evolutionary selection and for the mechanisms of inheritance of *PROKR2* L173R-associated GnRH deficiency.

The enigma: How can a founder mutation that impairs the neuroendocrine control of reproduction survive natural selection?

The persistence of founder mutations in the human gene pool despite their deleterious effects in the homozygous state is considered to be due to "overdominant" selection of heterozygous carriers over homozygous carriers and non-carriers. For example, *CFTR* F508del heterozygotes may be relatively protected compared to non-carriers against typhoid fever (40) and cholera (41). Interestingly, *PROKR2* L173R has a much lower carrier rate among unaffected subjects (0.16%) compared to other ancient founder mutations, such as *CFTR* F508del (cystic fibrosis; 4% in Northern Europe (39)), or *PAH* R408W [MIM 612349] (phenylketonuria [MIM 261600]; Neolithic era; 1% in Ireland (42)). The carrier rate of a founder mutation depends on its geographic and temporal origin, migration patterns, population bottlenecks, genetic drift, and particularly on the strength of positive selective pressure. Whether *PROKR2* L173R heterozygotes have a selective advantage (when not manifesting GnRH deficiency-associated infertility) is

presently unknown. Nevertheless, since prokineticins have been associated with several physiologic processes in addition to neurogenesis and reproduction in animal models, it is tempting to speculate that *PROKR2* heterozygotes have a selective advantage related to one or more of those functions (Table 3). However, evidence supporting such functions of prokineticins in humans is currently lacking. For example, whereas Prok2/Prokr2 signaling was shown to be involved in the regulation of energy balance in mice (43, 44), obesity was equally prevalent in GnRH-deficient patients with or without *PROKR2* mutations (16).

On the other hand, we recently reported *PROKR2* L173R mutation in a proband with hypothalamic amenorrhea (27), a reversible form of GnRH deficiency triggered by stressors, including energy deficits. We show here that this patient carries the founder mutation. Thus, the persistence of an allele that impairs reproduction may in fact relate to its value in suppressing fertility under certain conditions. During negative environmental circumstances of malnutrition such as famine, excessive energy expenditures during migration, and social stress during high predator density, being genetically predisposed to postpone the energetically costly process of reproduction could have been advantageous to both the female and her future offspring. Thus, the *PROKR2* L173R mutation may have conferred protection on female carriers by setting a lower threshold for inhibition of the reproductive neuroendocrine axis during periods when pregnancy would drain the bearer's nutritional and/or energy resources (27). The presented hypothesis on survival value of the mutation is currently lacking confirmation data as it targets genetic influences on survival in environmental conditions of ancient past. Identification of the same mutation in particular population subgroups with known

history of migration would permit further study of the hypothesis. Other mean of testing our speculation would be by using "knock-in" experiments in animal models exposed to environmental pressures, with the limitation of interspecies variation.

An exegesis: Congenital GnRH deficiency associated with *PROKR2* L173R is inherited in an oligogenic manner

The disease phenotype associated with *TAC3* c.209-1G>C, *GNRH1* c.18-19insA, and most other known founder mutations impairing reproduction, such as *CFTR* F508del, is inherited as a recessive trait (24, 25, 38, 45, 46). In contrast, congenital GnRH deficiency associated with *PROKR2* L173R does not appear to be inherited in a recessive fashion, as *PROKR2* L173R heterozygotes are likely to be GnRH-deficient and thus infertile (Table 1). Recent studies have shown that inheritance of GnRH deficiency associated with *PROKR2* L173R and other *PROKR2* mutations is complex and does not consistently conform to a monogenic pattern of inheritance (16, 17). Whereas biallelic *PROKR2* mutations show complete penetrance (16), most of the patients in this cohort have monoallelic mutations which display incomplete penetrance (Figure 3). Additional mutations in other genes associated with isolated GnRH deficiency, such as *PROK2*, *KAL1*, and *FGFR1*, have been documented to coexist in several patients with *PROKR2* mutations (including L173R) (11, 12, 15-17, 34). These findings indicate that GnRH deficiency associated with *PROKR2* L173R is inherited in an oligogenic manner and thus suggest it is unlikely that heterozygosity for the L173R mutation alone causes the full syndrome of severe GnRH deficiency and infertility. Rather, it appears that the spectrum

of reproductive phenotypes associated with *PROKR2* L173R, ranging from the common and reversible HA to the rare and severe GnRH deficiency, results from interaction of the mutation with either environmental (HA) or additional, yet to be identified, genetic defects.

In conclusion, *PROKR2* L173R is an ancient founder mutation that was not eliminated during evolution despite being associated with GnRH deficiency and failure of reproductive competency. Future studies should elucidate the factors involved in the selection process of the mutation which may include those affecting the penetrance of GnRH deficiency in heterozygotes such as oligogenicity (16, 17), the predisposition of carriers to become reproductively quiescent where such conservation could assist their survival (hypothalamic amenorrhea), and/or potential non-reproductive roles of the prokineticin system.

SUBJECTS, MATERIALS AND METHODS

Subjects

Haplotype mapping was performed in 22 unrelated probands with congenital GnRH deficiency carrying the *PROKR2* L173R mutation and 30 first-degree family members who were available from 12 pedigrees. The carrier rate and MAF of *PROKR2* L173R in GnRH-deficient patients and ethnically matched unaffected volunteers with normal puberty and reproductive function was evaluated in a total of 1,299 GnRH deficient patients with and 1214 unaffected controls: 1,048 patients and 729 controls from six published studies (11-13, 15-17) and an additional 251 patients and 485 controls

genotyped in this study. In addition, haplotype mapping was performed in 1 patient with HA carrying the *PROKR2* L173R (27) and her 3 first-degree family members. The ethics committees of participating institutions approved the study and informed consent was obtained from all subjects before enrollment.

Subject phenotyping

Idiopathic hypogonadotropic hypogonadism (IHH) was diagnosed according to standard criteria: (i.) serum testosterone (T) ≤100 ng/dl in men or estradiol (E2) ≤20 pg/ml in women, with low or normal serum gonadotropins; (ii.) otherwise normal anterior pituitary function; and (iii.) normal serum ferritin. A detailed individual and family history included pubertal development and associated reproductive and non-reproductive phenotypes, i.e., delayed puberty, anosmia, cryptorchidism, microphallus, synkinesia, obesity, hearing loss, ocular disorders, skeletal and renal malformations, and midline defects (cleft lip or palate and bifid uvula). A complete physical examination included Tanner staging and testicular volume measurement using a Prader orchidometer. The degree of pubertal development was based on clinical history and/or testicular size (47). Olfactory acuity was assessed by clinical history and, when possible, by olfactory smell testing (48). Imaging studies included renal ultrasound and MRI of the hypothalamo-pituitary region and olfactory bulbs.

The proband with hypothalamic amenorrhea has been described in detail in a prior report (27).

Gene sequencing

Genetic analysis of 10 genes underlying GnRH deficiency was performed using previously described gene sequencing methods. Exons and intron-exon junctions were analyzed for alterations in *PROKR2* (11) (in 23 *PROKR2* L173R carrying probands, additional 247 GnRH deficient probands and 185 controls) and other genes underlying GnRH deficiency: *KAL1* (49) (MIM 308700), *GNRHR* (18), *GNRH1* (38) (MIM 152760), *FGFR1* (50) (MIM 136350), *FGF8* (51) (MIM 600483), *PROK2* (9, 11), *KISS1R* (52) (MIM 604161), *TACR3* and *TAC3* (24) (MIM 162330) - all genes were sequenced in 13 probands, in 9 probands a subset of genes was sequenced and in one proband no additional gene beside *PROKR2* was analyzed. All sequence variations were identified on both strands, and mutations were confirmed in independent PCRs. Nucleotide and amino acid variations are described using standard nomenclature (53). To determine the familial segregation of identified mutations and delineate genotype-phenotype correlations, first-degree relatives available in the pedigrees of 13/23 haplotyped probands were genotyped; 4 of these pedigrees were previously reported (11, 27, 34).

Haplotype mapping

Two sets of polymorphic markers were genotyped in each proband and family member: short tandem repeats (STRs) and single nucleotide polymorphisms (SNPs). (i) Five STR markers flanking *PROKR2* were genotyped (Supplemental Table 1): *D20S849* and *D20S895* are respectively 89kb and 196kb telomeric to the L173R mutation, while *D20S835*, *D20S873* and *D20S882* are respectively 32kb, 313kb and 351kb centromeric. PCR amplification conditions and primer sequences used for STR marker amplification

are available upon request at the corresponding author. Forward primers were labeled with fluorescein (FAM). PCR product sizes were analyzed at the MGH DNA Core Lab using capillary electrophoresis on an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA), and amplicon sizes were determined using the ABI GeneMapper 4.0 (Applied Biosystems, Foster City, CA). (ii) Three exonic SNPs were genotyped by sequencing (11). In addition 95 tag SNPs in the region flanking *PROKR2* between STRs *D20S849* and *D20S882* (Supplemental Table 1) were selected from the HapMap CEU reference panel using the *Tagger* function in Haploview 4.1 (web URL: http://www.broadinstitute.org/haploview/haploview; Broad Institute, Cambridge, MA) (29). Tag SNP selection algorithm "pairwise tagging" (54) was used to capture SNPs of a MAF >0.001 with a correlation coefficient (r^2) higher than 0.6. SNP genotyping was performed in multiplex by The Broad Institute Center for Genotyping and Analysis using Sequenom MassARRAY iPLEX Gold (Sequenom, San Diego, CA) (55). A bead-less and label-free primer-extension chemistry was used with a high-fidelity polymerase to generate allele-specific products whose distinct masses were detected by MALDI-TOF MS (56). The genotypes of the multiplexed SNPs were differentiated using extension primers with unique masses. Two SNPs had extremely-low call rate and were therefore removed from further evaluation (rs7267662 and rs11699382). Five HapMap DNA samples were used as controls for the accuracy of genotyping. The haplotypes of the 13 probands with available first-degree relatives were determined from the genotypes of the SNPs and STR markers by considering the parental genotypes. Comparing these haplotypes, the presence of a shared haplotype was established and its size determined.

The frequency of the shared haplotype in the CEU population of the HapMap Project (web URL: http://hapmap.ncbi.nlm.nih.gov/) was obtained using Haploview 4.1 (29).

Nature and chronology of *PROKR2* L173R

The probability that *PROKR2* L173R is a hot spot mutation that arose *de novo* multiple independent times on the same haplotype was calculated using the binomial probability formula. The age of the mutation was estimated from 13 informative probands' mutation-carrying haplotypes by a previously described algorithm based on haplotype-decay models (30). The rationale is that the age of the mutation is inversely proportional to the size of the shared haplotype, because meiotic recombination over consecutive generations degrades the ancestral haplotype on which the mutation initially occurred (57). To decipher the history of mutation ancestry the shape of the genetic tree was considered, taking into account the density, length and position of branches on both sides of the mutation. The positions of branches in a mutation genetic tree are a reflection of both mutation-specific natural selection and background population history. Because various genetic trees could be behind the haplotypes obtained, specific algebraic expressions associated with any possible genealogical framework were constructed. Haplotype decay in any branch or stem is a power function or a compound of power functions, an example for a one-node five-branch tree being the following ("n" stands for the number of generations; "s" stands for the genetic interval):

$2n^4(1-s_1)^{n-1}(1-s_2)^{n-1}(1-s_3)^{n-1}(1-s_4)^{2n-1}$

For a two-node three-branch tree, the two complementary paths of construction are represented by the alternative equations, where "n" is the number of generations for the root node and "u" the number of generations for the branch node:

$2nu(1-s_1)^{u-1}(1-s_2)^{2n-1}$

$2u(2n-u)(1-s_1)^{2n-u-1}(1-s_2)^{2u-1}$

The method is recursive and the possible construction paths reflect the hierarchical organization of genealogical trees, because there are always several possible ways to break down a complex genealogy into subtrees.

For the age estimation an intergenerational time interval of 30 years was used (58).

SUPPLEMENTARY MATERIAL

A table of haplotypes is included in the supplementary material.

FUNDING

This work was supported by the National Institutes of Health [U54 HD028138 and R01 HD015788 to W.F.C., R01 HD056264 to N.P., P01 GM061354 to J.F.G.]; the Agence Nationale de la Recherche [ANR-09-GENO-017 to C.D.]; the Slovene National Research Agency [P3-0343 to M.A.]; and the National Center for Research Resources [U54 RR020278 to the Broad Institute Center for Genotyping and Analysis].

ACKNOWLEDGMENT

We are grateful to all patients and families for their kind participation.

CONFLICT OF INTEREST STATEMENT

The authors have nothing to disclose.

REFERENCES

1. Knobil, E. (1980) The neuroendocrine control of the menstrual cycle. *Recent. Prog. Horm. Res.*, **36**, 53-88.

2. Crowley, W. F., Jr., Filicori, M., Spratt, D. I. and Santoro, N. F. (1985) The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. *Recent. Prog. Horm. Res.*, **41**, 473-531.

3. Sykiotis, G. P., Pitteloud, N., Seminara, S. B., Kaiser, U. B. and Crowley, W. F., Jr. (2010) Deciphering genetic disease in the genomic era: the model of GnRH deficiency. *Sci. Transl. Med.*, **2**, 32rv32.

4. Seminara, S. B., Hayes, F. J. and Crowley, W. F., Jr. (1998) Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations. *Endocr. Rev.*, **19**, 521-539.

5. Martin, C., Balasubramanian, R., Dwyer, A. A., Au, M. G., Sidis, Y., Kaiser, U. B., Seminara, S. B., Pitteloud, N., Zhou, Q. Y. and Crowley, W. F., Jr. (2011) The role of the prokineticin 2 pathway in human reproduction: evidence from the study of human and murine gene mutations. *Endocr. Rev.*, **32**, 225-246.

6. Cheng, M. Y., Bullock, C. M., Li, C., Lee, A. G., Bermak, J. C., Belluzzi, J., Weaver, D. R., Leslie, F. M. and Zhou, Q. Y. (2002) Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature*, **417**, 405-410.

7. Cheng, M. Y., Leslie, F. M. and Zhou, Q. Y. (2006) Expression of prokineticins and their receptors in the adult mouse brain. *J. Comp. Neurol.*, **498**, 796-809.

8. Ng, K. L., Li, J. D., Cheng, M. Y., Leslie, F. M., Lee, A. G. and Zhou, Q. Y. (2005) Dependence of olfactory bulb neurogenesis on prokineticin 2 signaling. *Science*, **308**, 1923-1927.

9. Pitteloud, N., Zhang, C., Pignatelli, D., Li, J. D., Raivio, T., Cole, L. W., Plummer, L., Jacobson-Dickman, E. E., Mellon, P. L., Zhou, Q. Y., *et al.* (2007) Loss-of-function mutation in the prokineticin 2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proc. Natl. Acad. Sci. U S A*, **104**, 17447-17452.

10. Matsumoto, S., Yamazaki, C., Masumoto, K. H., Nagano, M., Naito, M., Soga, T., Hiyama, H., Matsumoto, M., Takasaki, J., Kamohara, M., *et al.* (2006) Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. *Proc. Natl. Acad. Sci. U S A*, **103**, 4140-4145.

11. Dode, C., Teixeira, L., Levilliers, J., Fouveaut, C., Bouchard, P., Kottler, M. L., Lespinasse, J., Lienhardt-Roussie, A., Mathieu, M., Moerman, A., *et al.* (2006) Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genet.*, **2**, e175.

12. Cole, L. W., Sidis, Y., Zhang, C., Quinton, R., Plummer, L., Pignatelli, D., Hughes, V. A., Dwyer, A. A., Raivio, T., Hayes, F. J., *et al.* (2008) Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. *J. Clin. Endocrinol. Metab.*, **93**, 3551-3559.

13. Abreu, A. P., Trarbach, E. B., de Castro, M., Frade Costa, E. M., Versiani, B., Matias Baptista, M. T., Garmes, H. M., Mendonca, B. B. and Latronico, A. C. (2008) Loss-of-function mutations in the genes encoding prokineticin-2 or prokineticin receptor-2 cause autosomal recessive Kallmann syndrome. *J. Clin. Endocrinol. Metab.*, **93**, 4113-4118.

14. Sinisi, A. A., Asci, R., Bellastella, G., Maione, L., Esposito, D., Elefante, A., De Bellis, A., Bellastella, A. and Iolascon, A. (2008) Homozygous mutation in the prokineticin-receptor2 gene (Val274Asp) presenting as reversible Kallmann syndrome and persistent oligozoospermia: case report. *Hum. Reprod.*, **23**, 2380-2384.

15. Canto, P., Munguia, P., Soderlund, D., Castro, J. J. and Mendez, J. P. (2009) Genetic analysis in patients with Kallmann syndrome: coexistence of mutations in prokineticin receptor 2 and KAL1. *J. Androl.*, **30**, 41-45.

16. Sarfati, J., Guiochon-Mantel, A., Rondard, P., Arnulf, I., Garcia-Pinero, A., Wolczynski, S., Brailly-Tabard, S., Bidet, M., Arroyo, R., Mathieu, M., *et al.* (2010) A Comparative Phenotypic Study of Kallmann Syndrome Patients Carrying Monoallelic and Biallelic Mutations in the Prokineticin 2 or Prokineticin Receptor 2 Genes. *J. Clin. Endocrinol. Metab.*, **95**, 659-669.

17. Sykiotis, G. P., Plummer, L., Hughes, V. A., Au, M., Durrani, S., Nayak-Young, S., Dwyer, A. A., Quinton, R., Hall, J. E., Gusella, J. F., *et al.* (2010) Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. *Proc. Natl. Acad. Sci. U S A*, **107**, 15140-15144.

18. de Roux, N., Young, J., Misrahi, M., Genet, R., Chanson, P., Schaison, G. and Milgrom, E. (1997) A family with hypogonadotropic hypogonadism and mutations in the gonadotropin-releasing hormone receptor. *N. Engl. J. Med.*, **337**, 1597-1602.

19. Layman, L. C., Cohen, D. P., Jin, M., Xie, J., Li, Z., Reindollar, R. H., Bolbolan, S., Bick, D. P., Sherins, R. R., Duck, L. W., *et al.* (1998) Mutations in gonadotropin-releasing hormone receptor gene cause hypogonadotropic hypogonadism. *Nat. Genet.*, **18**, 14-15.

20. Costa, E. M., Bedecarrats, G. Y., Mendonca, B. B., Arnhold, I. J., Kaiser, U. B. and Latronico, A. C. (2001) Two novel mutations in the gonadotropin-releasing hormone receptor gene in Brazilian patients with hypogonadotropic hypogonadism and normal olfaction. *J. Clin. Endocrinol. Metab.*, **86**, 2680-2686.

21. Bhagavath, B., Ozata, M., Ozdemir, I. C., Bolu, E., Bick, D. P., Sherins, R. J. and Layman, L. C. (2005) The prevalence of gonadotropin-releasing hormone receptor mutations in a large cohort of patients with hypogonadotropic hypogonadism. *Fertil. Steril.*, **84**, 951-957.

22. Cerrato, F., Shagoury, J., Kralickova, M., Dwyer, A., Falardeau, J., Ozata, M., Van Vliet, G., Bouloux, P., Hall, J. E., Hayes, F. J., *et al.* (2006) Coding sequence analysis of GNRHR and GPR54 in patients with congenital and adult-onset forms of hypogonadotropic hypogonadism. *Eur. J. Endocrinol.*, **155 Suppl 1**, S3-S10.

23. Kim, H. G., Pedersen-White, J., Bhagavath, B. and Layman, L. C. (2010) Genotype and Phenotype of Patients with Gonadotropin-Releasing Hormone Receptor Mutations. *Front. Horm. Res.*, **39**, 94-110.

24. Topaloglu, A. K., Reimann, F., Guclu, M., Yalin, A. S., Kotan, L. D., Porter, K. M., Serin, A., Mungan, N. O., Cook, J. R., Ozbek, M. N., *et al.* (2009) TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nat. Genet.*, **41**, 354-358.

25. Young, J., Bouligand, J., Francou, B., Raffin-Sanson, M. L., Gaillez, S., Jeanpierre, M., Grynberg, M., Kamenicky, P., Chanson, P., Brailly-Tabard, S., *et al.* (2010) TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. *J. Clin. Endocrinol. Metab.*, **95**, 2287-2295.

26. Gianetti, E., Tusset, C., Noel, S. D., Au, M. G., Dwyer, A. A., Hughes, V. A., Abreu, A. P., Carroll, J., Trarbach, E., Silveira, L. F., *et al.* (2010) TAC3/TACR3 Mutations Reveal Preferential Activation of Gonadotropin-Releasing Hormone

Release by Neurokinin B in Neonatal Life Followed by Reversal in Adulthood. *J. Clin. Endocrinol. Metab.*, **95**, 2857-2867.

27. Caronia, L., Martin, C., Sykiotis, G., Welt, C., Quinton, R., Avbelj, M., Plummer, L., Thambundit, A., Nayak-Young, S., Hughes, V., *et al.* (2011) A genetic basis for functional hypothalamic amenorrhea. *N. Engl. J. Med.*, **364**, 215-225.

28. Monnier, C., Dode, C., Fabre, L., Teixeira, L., Labesse, G., Pin, J. P., Hardelin, J. P. and Rondard, P. (2009) PROKR2 missense mutations associated with Kallmann syndrome impair receptor signalling activity. *Hum. Mol. Genet.*, **18**, 75-81.

29. Barrett, J. C., Fry, B., Maller, J. and Daly, M. J. (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*, **21**, 263-265.

30. Hanein, S., Perrault, I., Gerber, S., Delphin, N., Benezra, D., Shalev, S., Carmi, R., Feingold, J., Dufier, J. L., Munnich, A., *et al.* (2008) Population history and infrequent mutations: how old is a rare mutation? GUCY2D as a worked example. *Eur. J. Hum. Genet.*, **16**, 115-123.

31. Vagenakis, G. A., Sgourou, A., Papachatzopoulou, A., Kourounis, G., Papavassiliou, A. G. and Georgopoulos, N. A. (2005) The gonadotropin-releasing hormone (GnRH)-1 gene, the GnRH receptor gene, and their promoters in patients with idiopathic hypogonadotropic hypogonadism with or without resistance to GnRH action. *Fertil. Steril.*, **84**, 1762-1765.

32. Topaloglu, A. K., Lu, Z. L., Farooqi, I. S., Mungan, N. O., Yuksel, B., O'Rahilly, S. and Millar, R. P. (2006) Molecular genetic analysis of normosmic hypogonadotropic hypogonadism in a Turkish population: identification and detailed functional characterization of a novel mutation in the gonadotropin-releasing hormone receptor gene. *Neuroendocrinology*, **84**, 301-308.

33. Pitteloud, N., Quinton, R., Pearce, S., Raivio, T., Acierno, J., Dwyer, A., Plummer, L., Hughes, V., Seminara, S., Cheng, Y. Z., *et al.* (2007) Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. *J. Clin. Invest.*, **117**, 457-463.

34. Shaw, N. D., Seminara, S. B., Welt, C. K., Au, M. G., Plummer, L., Hughes, V. A., Dwyer, A. A., Martin, K. A., Quinton, R., Mericq, V., *et al.* (2011) Expanding the Phenotype and Genotype of Female GnRH Deficiency. *J. Clin. Endocrinol. Metab.*,

35. Nachtigall, L. B., Boepple, P. A., Pralong, F. P. and Crowley, W. F., Jr. (1997) Adult-onset idiopathic hypogonadotropic hypogonadism--a treatable form of male infertility. *N. Engl. J. Med.*, **336**, 410-415.

36. Schinzel, A., Lorda-Sanchez, I., Binkert, F., Carter, N. P., Bebb, C. E., Ferguson-Smith, M. A., Eiholzer, U., Zachmann, M. and Robinson, W. P. (1995) Kallmann syndrome in a boy with a t(1;10) translocation detected by reverse chromosome painting. *J. Med. Genet.*, **32**, 957-961.

37. Crow, J. F. and Kimura, M. (1979) Efficiency of truncation selection. *Proc. Natl. Acad. Sci. U S A*, **76**, 396-399.

38. Bouligand, J., Ghervan, C., Tello, J. A., Brailly-Tabard, S., Salenave, S., Chanson, P., Lombes, M., Millar, R. P., Guiochon-Mantel, A. and Young, J. (2009) Isolated familial hypogonadotropic hypogonadism and a GNRH1 mutation. *N. Engl. J. Med.*, **360**, 2742-2748.

39. Casals, T., Vazquez, C., Lazaro, C., Girbau, E., Gimenez, F. J. and Estivill, X. (1992) Cystic fibrosis in the Basque country: high frequency of mutation delta F508 in patients of Basque origin. *Am. J. Hum. Genet.*, **50**, 404-410.

40. Pier, G. B., Grout, M., Zaidi, T., Meluleni, G., Mueschenborn, S. S., Banting, G., Ratcliff, R., Evans, M. J. and Colledge, W. H. (1998) Salmonella typhi uses CFTR to enter intestinal epithelial cells. *Nature*, **393**, 79-82.

41. Hogenauer, C., Santa Ana, C. A., Porter, J. L., Millard, M., Gelfand, A., Rosenblatt, R. L., Prestidge, C. B. and Fordtran, J. S. (2000) Active intestinal chloride secretion in human carriers of cystic fibrosis mutations: an evaluation of the hypothesis that heterozygotes have subnormal active intestinal chloride secretion. *Am. J. Hum. Genet.*, **67**, 1422-1427.

42. Zschocke, J. (2003) Phenylketonuria mutations in Europe. *Hum. Mutat.*, **21**, 345-356.

43. Gardiner, J. V., Bataveljic, A., Patel, N. A., Bewick, G. A., Roy, D., Campbell, D., Greenwood, H. C., Murphy, K. G., Hameed, S., Jethwa, P. H., *et al.* (2009) Prokineticin 2 is a hypothalamic neuropeptide which potently inhibits food intake. *Diabetes*,

44. Jethwa, P. H., I'Anson, H., Warner, A., Prosser, H. M., Hastings, M. H., Maywood, E. S. and Ebling, F. J. (2008) Loss of prokineticin receptor 2 signaling predisposes mice to torpor. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **294**, R1968-1979.

45. Chan, Y. M., de Guillebon, A., Lang-Muritano, M., Plummer, L., Cerrato, F., Tsiaras, S., Gaspert, A., Lavoie, H. B., Wu, C. H., Crowley, W. F., Jr., *et al.* (2009) GNRH1 mutations in patients with idiopathic hypogonadotropic hypogonadism. *Proc. Natl. Acad. Sci. U S A*, **106**, 11703-11708.

46. Claustres, M., Guittard, C., Bozon, D., Chevalier, F., Verlingue, C., Ferec, C., Girodon, E., Cazeneuve, C., Bienvenu, T., Lalau, G., *et al.* (2000) Spectrum of CFTR mutations in cystic fibrosis and in congenital absence of the vas deferens in France. *Hum. Mutat.*, **16**, 143-156.

47. Pitteloud, N., Hayes, F. J., Boepple, P. A., DeCruz, S., Seminara, S. B., MacLaughlin, D. T. and Crowley, W. F., Jr. (2002) The role of prior pubertal development, biochemical markers of testicular maturation, and genetics in elucidating the phenotypic heterogeneity of idiopathic hypogonadotropic hypogonadism. *J. Clin. Endocrinol. Metab.*, **87**, 152-160.

48. Doty, R. L., Applebaum, S., Zusho, H. and Settle, R. G. (1985) Sex differences in odor identification ability: a cross-cultural analysis. *Neuropsychologia*, **23**, 667-672.

49. Hardelin, J. P., Levilliers, J., Blanchard, S., Carel, J. C., Leutenegger, M., Pinard-Bertelletto, J. P., Bouloux, P. and Petit, C. (1993) Heterogeneity in the mutations responsible for X chromosome-linked Kallmann syndrome. *Hum. Mol. Genet.*, **2**, 373-377.

50. Dode, C., Levilliers, J., Dupont, J. M., De Paepe, A., Le Du, N., Soussi-Yanicostas, N., Coimbra, R. S., Delmaghani, S., Compain-Nouaille, S., Baverel, F., *et al.* (2003) Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nat Genet*, **33**, 463-465.

51. Falardeau, J., Chung, W. C., Beenken, A., Raivio, T., Plummer, L., Sidis, Y., Jacobson-Dickman, E. E., Eliseenkova, A. V., Ma, J., Dwyer, A., *et al.* (2008) Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. *J. Clin. Invest.*, **118**, 2822-2831.

52. de Roux, N., Genin, E., Carel, J. C., Matsuda, F., Chaussain, J. L. and Milgrom, E. (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. U S A*, **100**, 10972-10976.

53. den Dunnen, J. T. and Antonarakis, S. E. (2001) Nomenclature for the description of human sequence variations. *Hum. Genet.*, **109**, 121-124.

54. de Bakker, P. I., Yelensky, R., Pe'er, I., Gabriel, S. B., Daly, M. J. and Altshuler, D. (2005) Efficiency and power in genetic association studies. *Nat. Genet.*, **37**, 1217-1223.

55. Gabriel, S., Ziaugra, L. and Tabbaa, D. (2009) SNP genotyping using the Sequenom MassARRAY iPLEX platform. *Curr. Protoc. Hum. Genet.*, **Chapter 2**, Unit 2 12.

56. Storm, N., Darnhofer-Patel, B., van den Boom, D. and Rodi, C. P. (2003) MALDI-TOF mass spectrometry-based SNP genotyping. *Methods. Mol. Biol.*, **212**, 241-262.

57. McPeek, M. S. and Strahs, A. (1999) Assessment of linkage disequilibrium by the decay of haplotype sharing, with application to fine-scale genetic mapping. *Am. J. Hum. Genet.*, **65**, 858-875.

58. Tremblay, M. and Vezina, H. (2000) New estimates of intergenerational time intervals for the calculation of age and origins of mutations. *Am. J. Hum. Genet.*, **66**, 651-658.

59. Shojaei, F., Wu, X., Zhong, C., Yu, L., Liang, X. H., Yao, J., Blanchard, D., Bais, C., Peale, F. V., van Bruggen, N., *et al.* (2007) Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature*, **450**, 825-831.

60. Shojaei, F., Singh, M., Thompson, J. D. and Ferrara, N. (2008) Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc. Natl. Acad. Sci. U S A*, **105**, 2640-2645.

61. Monnier, J. and Samson, M. (2008) Cytokine properties of prokineticins. *FEBS J.*, **275**, 4014-4021.

62. Li, J. D., Hu, W. P. and Zhou, Q. Y. (2009) Disruption of the circadian output molecule prokineticin 2 results in anxiolytic and antidepressant-like effects in mice. *Neuropsychopharmacology*, **34**, 367-373.

LEGENDS TO FIGURES

Figure 1: Haplotypes of informative probands

For each STR marker, the number of dinucleotide repeats is shown. n.i. (not informative): alleles that cannot be unambiguously attributed to the L173R haplotype. Gray rectangle: region genotyped via SNP array analysis. Pink horizontal lines: haplotypes. Blue horizontal lines: haplotype regions identical to each other. In each of the homozygous probands (#2 and #12) the two haplotypes are labeled "a" and "b". The haplotype shared by all probands is marked with vertical dotted lines and is delimited by two major recombination hot spots on chromosome 20 (genomic region: NCBI build 36.1; recombination rates: HapMap, CEU population, public release #27).

Figure 2: Haplotypes in the HA pedigree

Red arrow: proband. Haplotypes in the HA pedigree are shown with representative polymorphisms and the *PROKR2* L173R mutation (c.518T>G). The mutation-bearing L173R haplotype is boxed red. SNPs and the mutation are stated according to the cDNA sequence, which is reverse and complementary to the genomic sequence.

Figure 3: Informative previously unpublished pedigrees

Red circles: probands. Red numbers: probands' IDs. Haplotypes in Pedigree #3 are presented with representative polymorphisms and the *PROKR2* L173R mutation (c.518T>G). The mutation-bearing L173R haplotype is boxed red. Black arrows indicate two siblings that have the same paternal and maternal *PROKR2* haplotypes, yet are

discordant for the disease. SNPs and the mutation are stated according to the cDNA sequence, which is reverse and complementary to the genomic.

TABLES

Table 1. *PROKR2* L173R mutation identified in patients and healthy controls.

Ethnicity	GnRH-Deficient Patients			Healthy Control Subjects		
	Number screened	Number of L173R carriers (homo/heterozygous)	MAF (%)	Number screened	Number of L173R carriers (homo/heterozygous)	MAF (%)
Caucasian	1,086	23 (1/22)	1.10	764	2 (0/2)	0.13
Brazilian	109	4 (0/4)	1.87	100	0	0
Mexican	24	2 (0/2)	4.17	200	0	0
Maghrebi	80	2 (1/1)	1.87	150	0	0
Total	**1,299**	**31 (2/29)**	**1.27**	**1214**	**2 (0/2)**	**0.08**

Key: MAF= minor allele frequency.

Table 2. Demographic and phenotypic characteristics of GnRH-deficient patients carrying the *PROKR2* L173R mutation.

Subject (Ref)	Sex	Ethnicity (origin)	Dx	Inheritance	Puberty	C/M	MRI	Additional phenotypes	*PROKR2*&Other gene defects[A]
1 (16)	M	Maghrebian (Morocco)	KS	Familial	Absent	-/-	Hypoplastic OB	/	L173R/WT
2 (16)	M	Maghrebian (Morocco)	KS	Sporadic	Absent	+/+	Aplastic OB	↑BW	L173R/L173R
3 (17)	F	Caucasian (Lebanon)	nIHH	Familial	Partial	/	NA	Aortic insuficiency	L173R/WT
4 (17)	F	Caucasian (Greece)	KS	Sporadic	Partial	/	Aplastic OB, partial empty sella	High arched palate, scoliosis	[M64V+L173R]/WT
5 (36)	M	Caucasian (Spain)	KS	Sporadic	Absent	+/-	Aplastic L OB, hypoplastic R OB	High arched palate, pes planus	L173R/WT der(1),t(1,10)(q44,q26) translocation[B]
6 (27)	F	Caucasian (UK)	HA	Familial	Normal	/	NA	Eating disorder	L173R/WT
7 (12)	M	Caucasian (UK)	KS	Sporadic	Absent	+/-	NA	2 supernumerary teeth	L173R/WT
8 (17)	M	Caucasian (UK)	KS	Sporadic	Absent	+/+	NA	Synkinesia, hypoplastic R ear, delayed motor development, delayed permanent tooth eruption	L173R/WT
9 (11),(16)	M	Caucasian (Europe)	KS	Familial	Absent	-/-	Aplastic OB	High arched palate, psychomotor problems, ↓BW	L173R/WT
10 (11),(16)	M	Caucasian (Europe)	KS	Familial	Absent	+/+	NA	/	L173R/Q210R
11 (34)	F	Caucasian (Europe)	KS	Familial	Absent	/	Aplastic R OB, hypoplastic L OB & sulcus	Diastema, cleft uvula, scoliosis, learning disabilities	L173R/WT *FGFR1* C55fsX45/WT

12 (11),(16)	M	Caucasian (Europe)	KS	Sporadic	Absent	+/+	Aplastic OB	/	L173R/L173R
13 (12)	M	Ashkenazi Jewish (Poland)	KS	Sporadic	Absent	-/-	Hypoplastic R olfactory sulcus, atrophic infundibulum	Synkinesia, pectus excavatum, pes planus, ↑BW	L173R/WT
14 (16)	M	Caucasian (Europe)	KS	Familial	Absent	+/-	Hypoplastic OB	Brachidactyly (hands), polydactyly (R foot)	L173R/WT
15	F	Caucasian (Europe)	nIHH	Sporadic	Absent	/	Partial empty sella	/	L173R/WT
16 (11),(16)	M	Caucasian (Europe)	KS	Sporadic	Absent	-/+	NA	↑BW	L173R/WT
17 (11),(16)	M	Caucasian (Europe)	KS	Sporadic	Absent	-/-	NA	Severe depression	L173R/WT
18 (16)	M	Caucasian (Europe)	KS	Sporadic	Absent	-/+	Normal OB	↓BW	L173R/WT
19 (17)	M	Caucasian (French Canada)	KS	Sporadic	Absent	-/-	Hypoplastic R OB, pituitary hypoplasia	Night blindness, ↑BW	L173R/WT
20 (13)	F	Brazilian (Brazil)	KS	Sporadic	Absent	/	Hypoplastic OB & sulci	↑BW	L173R/WT
21 (13)	M	Brazilian (Brazil)	KS	Sporadic	Absent	-/-	Aplastic OB & sulci	↑BW	L173R/WT
22	M	Brazilian (Brazil)	AHH	Sporadic	Normal	-/-	Normal OB	/	L173R/WT
23	M	Brazilian (Brazil)	nIHH	Sporadic	NA	-/-	NA	/	L173R/WT

Key: KS=Kallmann syndrome; nIHH=normosmic hypogonadotropic hypogonadism; HA=hypothalamic amenorrhea; AHH=adult onset GnRH deficiency; C=cryptorchidism; M=microphallus; (+)=present; (-)=absent; OB=olfactory bulb; R=right; L=left; NA=not assessed; BW=body weight; WT=wild type; The origin describes the ethnic background of the parents; [A] *KAL1*, *GNRHR*, *GNRH1*, *FGFR1*, *FGF8*, *PROK2*, *KISS1R*, *TAC3* and *TACR3* were sequenced in all probands except #5, #8 and #19 (all genes except *TAC3*, *TACR3* and *GNRH1*), #6 and #7 (all genes except *TAC3*, *TACR3*, *GNRH1* and *GNRHR*),

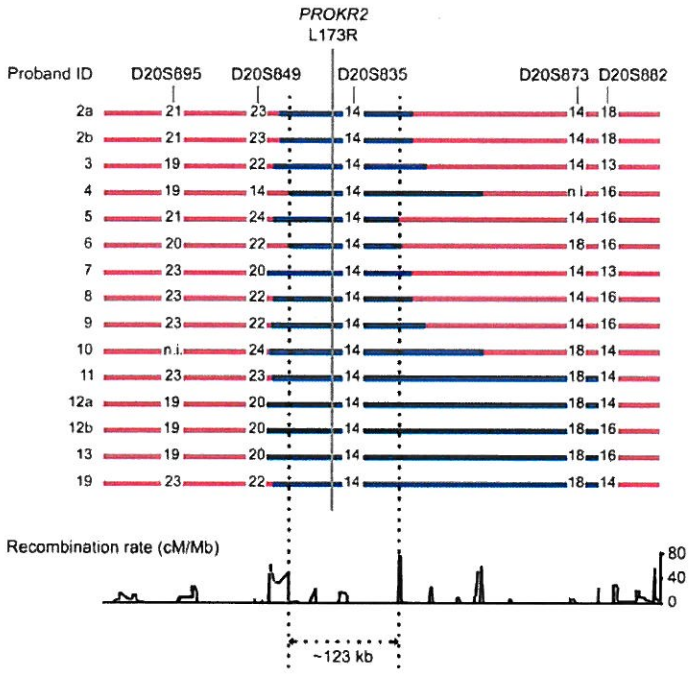
#13 (all genes except *TAC3* and *TACR3*), #20 (only *FGFR1*), #21 (only *KAL1* and *FGFR1*), #22 (no additional genes), #23 (only *KAL1*, *FGFR1*, and *GPR54*); [B] a *de novo* chromosomal rearrangement (36) whose implication for GnRH deficiency or prokineticin signaling is unclear.

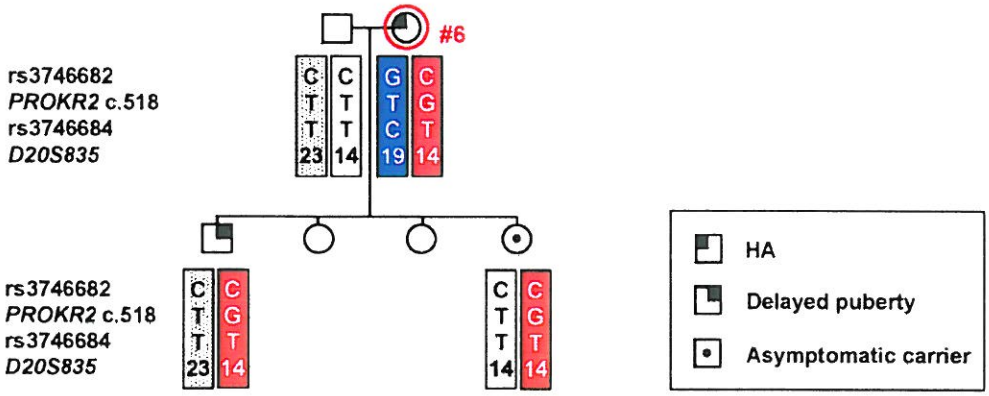
Table 3. Potential selective advantages of *PROKR2* L173R heterozygosity according to the non-reproductive functions of prokineticin signaling in model organisms.

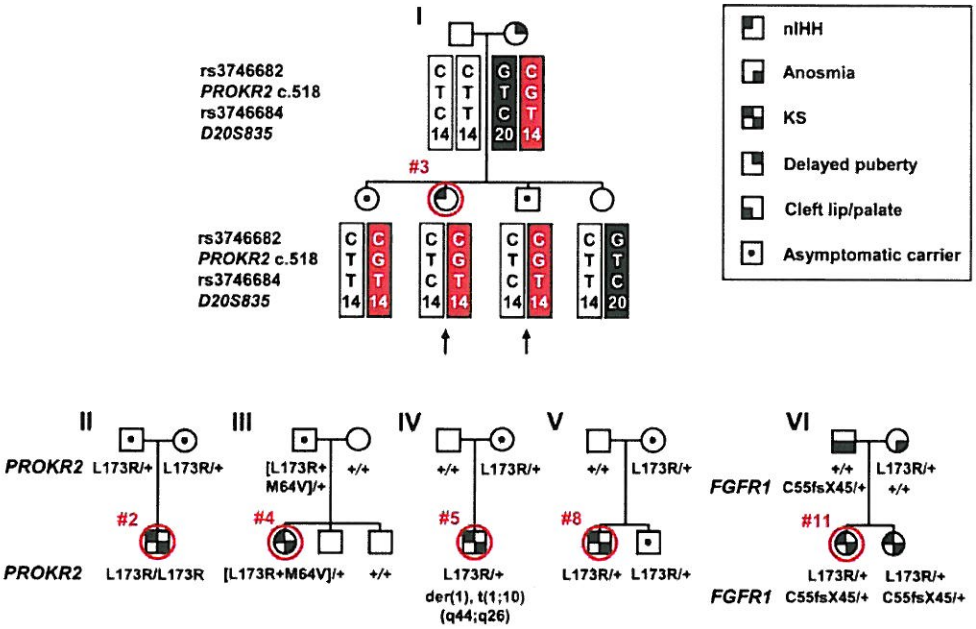
Function of Prok2/Prokr2 signaling	Ref.	Potential heterozygote advantage
Pro-inflammatory signaling that promotes tumor angiogenesis	(59, 60)	Decreased cancer risk
Modulation of immunity by induction of granulocyte and monocyte proliferation, chemotaxis, differentiation, survival and activation.	(61)	Resistance to infection and/or autoimmunity
Regulation of anxiety and depression-related behaviors	(62)	Improved stress coping abilities
Hypothalamic regulation of appetite and energy expenditure	(43, 44)	Adaptation to caloric restriction during famine

ABBREVIATIONS

GnRH, gonadotropin-releasing hormone; PROKR2, prokineticin receptor 2; PROK2, prokineticin 2; IHH, idiopathic hypogonadotropic hypogonadism; nIHH, normosmic idiopathic hypogonadotropic hypogonadism; KS, Kallmann syndrome; HA, hypothalamic amenorrhea; MAF, minor allele frequency; STR, short tandem repeat; SNP, single nucleotide polymorphism; *KAL1*, Kallmann syndrome 1 sequence gene, *GNRHR*, gonadotropin-releasing hormone receptor gene; *GNRH1*, gonadotropin-releasing hormone 1 gene; *FGFR1*, fibroblast growth factor receptor 1 gene; *FGF8*, fibroblast growth factor 8 gene; *KISS1R*, KISS1 receptor gene; *TACR3*, tachykinin receptor 3 gene; *TAC3*, tachykinin 3 gene.


PROKR2
L173R
Proband ID
D20S895
D20S849
D20S835
D20S873
D20S882
2a 21 23 14 14 18
2b 21 23 14 14 18
3 19 22 14 14 13
4 19 14 14 n.i. 16
5 21 24 14 14 16
6 20 22 14 18 16
7 23 20 14 14 13
8 23 22 14 14 16
9 23 22 14 14 16
10 n.i. 24 14 18 14
11 23 23 14 18 14
12a 19 20 14 18 14
12b 19 20 14 18 16
13 19 20 14 18 16
19 23 22 14 18 14
Recombination rate (cM/Mb)
80
40
0
~123 kb


#6
rs3746682
PROKR2 c.518
rs3746684
D20S835
C T T 23
C T T 14
G T C 19
C G T 14
rs3746682
PROKR2 c.518
rs3746684
D20S835
C T T 23
C G T 14
C T T 14
C G T 14
HA
Delayed puberty
Asymptomatic carrier


I
rs3746682
PROKR2 c.518
rs3746684
D20S835
CTC 14
CTT 14
GTC 20
CGT 14
#3
rs3746682
PROKR2 c.518
rs3746684
D20S835
CTT 14
CGT 14
CTC 14
CGT 14
CTC 14
CGT 14
CTT 14
GTC 20
nlHH
Anosmia
KS
Delayed puberty
Cleft lip/palate
Asymptomatic carrier
II
PROKR2
L173R/+
L173R/+
#2
PROKR2
L173R/L173R
III
[L173R+ M64V]/+
+/+
#4
[L173R+M64V]/+
+/+
IV
+/+
L173R/+
#5
L173R/+
der(1), t(1;10) (q44;q26)
V
+/+
L173R/+
#8
L173R/+
L173R/+
VI
FGFR1
+/+
C55fsX45/+
L173R/+
+/+
#11
L173R/+
C55fsX45/+
L173R/+
C55fsX45/+